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DEPARTMENT OF THE ARMY  
Fort Detrick  
Frederick, Maryland

FLUORESCENCE MICROSCOPE OBSERVATIONS OF  
TRICHOMONAS VAGINALIS  
TREATED WITH ACRIDINE ORANGE

[Following is a translation of an article by U. Fabio, R. Olivo and W. Pagnotta of the Institute of Hygiene of Modena University, Italy, in the Italian-language periodical, Nuovi Annali d'Igiene e Microbiologia (New Annals of Hygiene and Microbiology) Vol. 13, No 4, 1962, pages 274-278.]

Within the framework of a series of investigations into the biology of *Trichomonas vaginalis* and on the epidemiology of the infection induced by it, we directed our attention to the problem of finding a way to stain the living parasite so advantage could be taken of the possibilities offered by fluorescence microscopy.

From what we have been able to discover, the literature on the use of fluorescent agents in practically deficient in respect to *Trichomonas vaginalis*. It seems that no particular consideration has ever been given to the appearance of the live flagellate under ultraviolet light after it has been treated with fluorescent agents. Research carried out by Coutts and Silva-Inzunza (1954) dealing with vital staining of the flagellate with fluorescein, to be used for observations with a microscope under normal light or for dark field observations and research by Bertalanffy (1960, 1961) giving a marginal description of the appearance of the parasite in smears which had been fixed and treated with acridine orange, both dealt with investigations having other purposes and using different methods.

It therefore seemed to us worthwhile to investigate the flagellate by treating it with fluorochromes and then placing it under ultraviolet light for observation in the live state.

An entire series of such fluorochromes was used. Some brought out marked fluorescence in *Trichomonas vaginalis*; others produced a barely perceptible trace of fluorescence and others proved entirely inactive. We intend to report the overall results of these observations in a subsequent paper. This paper will be limited to reporting in detail the results obtained using acridine orange.

Acridine orange (3,6-tetramethyldiaminoacridine) has been used in biology as a fluorochrome which could be used for vital staining of plant cells and was used for this purpose for the first time by Bukatsch and Haitinger (1940) and by Strugger (1940), who also described a characteristic chromatic differentiation between live and dead cells brought about by the fluorochrome, itself. Since that time there has been a continuous increase in the number of times reference has been made to this method of staining in the literature, as this method also has shown itself to have but very low toxicity for live protoplasm.

#### PROCEDURE AND MATERIALS USED

Our procedure consisted in adding varying concentrations of acridine orange from 1:1000 to 1:500,000 to *Trichomonas vaginalis* cultures which had developed for 48 to 96 hours.

We used FE and GR strains of *Trichomonas vaginalis* isolated during the course of epidemiological research in the vicinity of Modena, Italy (Bartolotti, Fabio and Oppo, 1961). These strains had been preserved for some time in the laboratory in artificial cultures.

Trichosel broth was used as the culture substrate together with the standard-formula CPLM medium in the form which is free of methylene blue and free of agar.

Contact between the protozoan and the stain was prolonged for periods varying between a few minutes and 24 hrs., but always in the dark at 37°C.

pH readings of the suspensions examined showed variations between 5 and 5.30.

A Reichert Zetopan microscope equipped with fluorescence apparatus (Binolux illuminator system) with Osram HBO 200 W. mercury-vapor burner was used for the observations.

The preparations were observed using an E3 exciter filter which transmits the blue and ultraviolet components, with ocular coupling of the darkfield stop (Sp3).

### RESULTS AND DISCUSSION

We observed forms of the protozoan which showed greater or lesser degrees of motility and others which remained immobile.

The forms in motion showed brilliant green coloration of the cytoplasm and of the flagella, with a clearly evident nucleus having a lighter coloration and sometimes with cytoplasmatic granulations having a red-orange color (Fig. 1, Nos. 1, 2 and 3 and Fig. 2, No. 1).

The immobile forms, instead, stood out as a result of a red-orange to copper-red coloration of the cytoplasm with a yellow nucleus of varying brightness (Fig. 1, No. 4 and Fig. 2, Nos. 2 and 3).

The best results for purposes of observation were obtained by us under the following experimental conditions:

1. Acridine orange solutions between 1:4000 and 1:16000
2. Contact between the protozoan and the fluorochrome for from 1 to 6 hrs.

The two parameters (dilution of the fluorochrome and time of contact) showed themselves within certain limits to be correlated to one another by inverse and constant ratios.

The very low toxicity shown by this stain in respect to the *Trichomonas vaginalis* cultures should be emphasized once more as these organisms remain practically unchanged both morphologically and biologically. Almost identically matching results were obtained using Trichosel and both normal and modified CPLM cultures.

Prolonged observations of motile subjects carried on for a certain period of time with such subjects being subjected to ultraviolet radiation were particularly interesting.

We were able to observe with the passage of only a few minutes that these protozoans underwent a progressive change in color which ended in their death. Progressively as their translatory motions showed a tendency to slow down and as the action of the flagella and of the undulatory membrane became more and more sluggish, granulations appeared in the cytoplasm or those already present increased in size, being yellow at first and then red-orange with a tendency to cluster and to invade the entire body of the protozoan, with the exception of the nucleus. Sometimes the nucleus retained its own green color, turning color at a later time and going through a range of yellow shades different in intensity from the remainder of the protoplasm (Fig. 2, Nos. 4, 5, 6, 7, 8 and 9).

The phenomenon described above shows remarkable similarity to that reported for the first time by Strugger (1940, 1941, 1942, 1949) on live plant cells and then defined as the "thickening effect" (effetto di concentrazione).

This author, in a detailed analysis of the use of acridine orange in vital staining of cell parts had pointed out the considerable affinity which this substance has for protoplasm, its harmlessness in respect to the organism being stained and, above all, the characteristic color differentiation between live and dead cells. These observations were confirmed by Bukatsch (1941) and later by Höfler (1947) and Kolbel (1947).

The various authors who have interested themselves in this problem do not agree unanimously as to the interpretation of the phenomenon.

We believe the theory proposed by Strugger to be reliable, in that, starting from the observation that varying degrees of concentration of acridine orange solutions showed characteristic changes in the fluorescence spectrum, he suggested the hypothesis that the different coloration of the protoplasm in different stages was chiefly to be attributed to an actual "thickening effect" of the fluorochrome in the intracellular space. Developing this theory, the author specified further that the leaf-green color characteristic of the live protoplasm would correspond to an approximate concentration of the intracellular stain of 1:50,000, while the orange coloration of the dead cells would be caused by a greater accumulation, equal to about 1 per cent. Various factors (contact time, concentration of the stain, isoelectric point of the cytoplasm, etc.) would be held to interfere in various ways with the manifestation of the phenomenon. The age of the cell part treated with acridine orange would be held to variously affect the fluorescence induced by the

ultraviolet, such as happens with other fluorochemicals (Nau-mova 1960) and even the pH would be held to play a part of no small significance.

It is evident that the possibility of manifestation of the phenomenon must necessarily be also bound up with certain cyto-chemical affinities and therefore it cannot be said that it takes place in all cell parts, whether of animal or vegetable origin. This could also explain the negative results reported by some authors at times. Scarpa (1962), for example, in research on the differential coloration of certain schizomycetes using acridine orange did not find any possibility of obtaining any difference in coloration between live and dead parts and, furthermore, was unable to confirm the existence of any importance associated with the pH of the stain solution, with the gram-resistance of the microbe being tested, with the composition of the culture medium, nor even with the duration of contact between the microbe and the fluorochemical and the age of the culture.

#### CONCLUSIONS

In conclusion, we were able to obtain a coloration of *Trichomonas vaginalis* which would distinguish between live and dead cells, using a acridine orange in suitable solution and for proper contact times, our results being similar to those obtained by various authors for other live organisms.

The different fluorescences induced in the different stages, whose extremes are represented by a more or less intensely green cytoplasm typical of those forms showing intense motor activity and a yellow-orange, more or less marked coloration of the immobile forms and those about to decompose, make it possible to follow under the microscope the degeneration of the parasite under the effect of a harmful stimulus, such as is represented by ultraviolet rays, correlating the change in color to such degenerative changes.

We would therefore emphasize the interest attendant to the observations which have been made, also in respect to the potentialities for other applications in morpho-biological studies of protozoa in general.

#### SUMMARY

Fluorescence microscope observations of *Trichomonas vaginalis* treated in culture media with a acridine orange solution showed a different coloration between those organisms which were live and motile on the one hand and those which were immobile or undergoing degenerative changes. It also made it possible to follow up the changes in color in protozoa which were subjected to sustained exposure to ultraviolet radiation for some time.

GRAPHIC NOT REPRODUCIBLE

Fig. 1 - The GR strain of *Trichomonas vaginalis* grown for 53 hrs. in a Trichosel broth culture medium. Acridine orange diluted 1:8000. Contact time between the protozoan and the fluorochrome 3 hrs. Microphotographs made using flat Kodak Ektachrome film, daylight type, 18 Din/50 ASA. Exposure time: 30-40 Sec.

1 - 2 Forms of the protozoan in motile activity with clearly visible nuclei. 430X.

3 - Protozoans undergoing cell-division. 280X.

4 - Immobile forms with light-yellow nuclei and cytoplasm having orange clusters. 280X.



## GRAPHIC NOT REPRODUCIBLE

Fig. 2 1 - Motile forms with clearly visible nuclei. GR strain in Trichosel broth culture medium. Grown 72 hrs. Acridine orange diluted 1:4000. Contact time between the protozoan and the fluorochrome: 6 hrs. 110X.

2-3 Immobile forms (yellow nucleus and orange cytoplasm) and motile (green cytoplasm and nucleus with lighter shade). GR strain in Trichosel broth culture medium. Culture age: 72 hrs. Dilution 1:4000. Contact time protozoan-fluorochrome 8 hrs. 110X.

4-5-6 - Organisms in motion (green cytoplasm and nucleus with lighter shade); immobile forms or those undergoing degeneration (cytoplasm with orange or copper-red granulations or clusters and yellow nuclei). GR strains in Trichosel broth culture. Culture age: 72-96 hrs. Dilution 1:8000. Contact time protozoan-fluorochrome 1 to 6 hrs. 300X.

7-8 - Immobile protozoans. GR strain in CPLM culture medium. Culture age: 48-72 hrs. Dilutions 1:4000 to 1:16000. Contact: 2 hrs. 480X.

9 - *Trichomonas vaginalis* undergoing degeneration. The yellow nucleus can still be clearly seen, while the cytoplasm shows copper-red granulations. GR strain in CPLM culture medium. Culture age 3 hrs. Dilution 1:4000. Contact 2 hrs. 480X.

Microphotographs made using 24x36 mm. Kodak High-Speed Ektachrome film, Daylight Type 23 Din/160 ASA. Exposure time from 30 to 90 sec.

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